Identification of *Taxus cuspidata* Sieb. et Zucc. endophytic fungi—new species, species known and their metabolite

XIANG Yong[1], LU An-guo[2], WU Wen-fang[2]
[1] *Shenyang Pharmaceutical University, Shenyang 110016, P. R. China*
[2] *Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, P. R. China*

Abstract: A total of 94 isolates of endophytic fungi were isolated from the bark of 200-yr.-old *Taxus cuspidata* Sieb. et Zucc.In the primeval forest of the Changbai Mountain Natural Reserve, and 19 species of endophytic fungi were identified, including 10 new recorded-genus-species, 2 new species (*Phomopsis longiscoleosporu* Y. Xiang et Lu An Guo Wu Wen Fang, *Coniothyrium macrospoum* Y. Xiang J.X. et Lu An Guo Wu Wen Fang), 1 new varied species (*Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo) and 6 known species of China (*Eurotium amstelodomi* Mgngin, *Eurotium repens* de Bary, *Botrytis* sp., *Penicillium citrinum* Thom, *Epicoccum nigrium* LinK, *Fusarium* sp.). Through thin layer chromatography (TLC), liquid fermentation metabolite of the strains was determined, and four strains (*Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo Wu Wen Fang, *Botrytis* sp., *Eurotium amsteloodomi* Mgngin, *Eurotium repens* de Bary) were screened out, whose metabolites reacted positively with the vanillic aldehyde that was one special taxoid developer. Among the four strains, *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo, produced one compound largely, which positively reacted with one alkaloids developer—Bismuth potassium iodide. The compound is identified as taxoids type through spectrum analysis. This demonstrates that *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo can highly produce taxoids largely.

Keywords: *Taxus*; Endophytic fungi; Identification; Taxoids
CLC number: S432.44 **Document code**: A **Article ID**: 1007-662X(2003)04-0290-05

Introduction

Endophytic fungi are widely found in almost all kinds of plants, and their species composition and numbers are affected by ages of plants, environment factors, etc. Many endophytic fungi can produce some physiological active compounds, which are same or analogy with those isolated from their hosts. The novel anticancer medicine Taxol is a physiological active compound isolated from the bark of *Taxus*, but it is difficult to be extracted significantly owing to little content of physiological active compound in the bark of slowly growing *Taxus*. Recent studies showed that many endophytic fungi living in plants of *Taxus*, including *Taxus brevifolia* Nutt., *T. yunnanesis* Che et L.K. Fu, *T. wallichiann* Zucc., could produce taxol (Fisher *et al.* 1993; Helander *et al.* 1993; Strobel *et al.* 1993; Strobel *et al.* 1996; Guo 2001; Xiang 2002, 2003). This gives a new method in resolving resource limitation and searching alternative taxol source. Unfortunately, the yield of taxol is too low to achieve production in industrial level. In this paper, many endophytic fungi isolated from the bark of over 200-year-old *Taxus cuspidata* Sieb. et Zucc. in the primeval forest of the Changbai Mountain Natural Reserve are identified for determining their taxons, based on isolation and screening of *Taxus cuspidata*. endophytic fungi (Xiang 2002).

Biography: XIANG Yong (1959-), male, Associate professor in Shenyang Pharmaceutical University, Shenyang 110016, P. R. China.
Received date: 2003-09-02
Responsible editor: Zhu Hong

Materials and methods

Sources of *Taxus* specimens

Isolation specimens were collected from *Taxus cuspidata* Sieb. et Zucc. in the primeval forest of the Changbai Mountain Natural Reserve. From 1996 to 2000, the bark specimens of different tissues of *T. cuspidata*. were collected, numbered and reserved in paper bags.

Media

The following media were prepared and used in the experiment.

Mediasolid-state media

(1) PDA: 200-g potato, 20-g glucose, 20-g agar, and 1000-mL distilled water, with natural pH. Sterilization was carried out at 121 °C for 20 min.

(2) Ciaolk's Agar: 30-g sucrose, 3-g $NaNO_3$, 0.5-g KCl, 0.01-g $Fe_2(SO_4)_3$, 0.5-g $MgSO_4$, 1-g KH_2PO_4, 20-g agar and 1000-mL distilled water, with natural pH, sterilized at 121°C for 20 min.

(3) Corn meal-Sucrose Agar: 40-g Corn meal, 10-g sucrose, 20-g agar and 1000-mL distilled water, with natural pH, sterilized at 121°C for 20 min.

Liquid production media

(1) Seed medium was prepared with glucose (20 g), peanut (10 g), powder (10 g), $MgSO_4$ (3 g), KH_2PO_4 (3 g),

NH_4Cl (3 g) and distilled water of 1000 mL, with natural pH, and sterilized at 121 °C for 20 min.

(2) Production medium: glucose (10 g), peanut powder (10 g), soybean powder(10 g), starch (10 g), $MgSO_4$ (3 g), KH_2PO_4 (3 g), NH_4Cl (3 g) and distilled water of 1000 mL, with natural pH, were used and sterilized at 121 °C for 20 min.

Methods

Isolation and identification of endophytic fungi

(1) Isolation: The specimens of *T. cuspidata* were dipped in 75% ethanol for 1 min, and flamed on burning alcohol for 15 s. The inner section excised surface layer was put on the PDA solid media with antibiotic, such as, 100 μg/mL penicillin or 40 μg/mL streptomycin, or on the other solid media in Petri dishes, and incubated at 25 °C in the dark for some days. The tip of fungal hypha growing out from the inner bark was incubated at 25 °C and cultured onto the fresh PDA in culture tube. After several times of repeated subculture, the isolates were obtained.

(2) Identification: The ontogeny characteristics of hypha, conidiogenous structure, conidia during the culture were observed and described through direct-pick processing and slide-insert culture (Fang 1979). The genus and the species of the endophtic fungi were discerned, based on the literature retrieving (Barnett *et al.* 1972; Ainsworth *et al.* 1973; Von Arx. 1974).

Screening and structure identification of fermentation metabolite

The isolates were screened through TLC method. Target strains were screened by three following methods: i) the thin layer slates were directly examined under UV lights with 253.7 nm and 376.5nm; ii) NO.1 developer vanillic aldehyde was sprayed on the thin layer slates, then the thin layer slates were heated at 100 °C for 8 min; iii) Developer dilute potassium bismuth iodide was sprayed on the thin layer slates. The fermentation metabolites were extracted, separated, and purified by the methods of solvent extraction, TLC and column chromatography respectively. The structures of the metabolites were detected by methods of Ultraviolet scanning, Infrared scanning, MS and NMR (Xiang 2002, 2003).

Results

Isolation and identification of strains

A total of 94 isolates were isolated from the bark of *Taxus cuspidata* and 19 species were identified, composed of 2 novel species, 1 novel varied species, 6 species known in China and 10 novel recorded-species in China.

Description of the new species

(1) *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo var. nov.

Characteristics: Colonies cultured on PDA at 25-28 °C, were round and wooly. The mycelium was dense and massive and extended rapidly, and then turned initially white to grayness in colors. Culture medium gradually turned to blackish green and substratum turned black. Hyphae turned from buff to brown in colors, with septum. Conidiophores had the color from buff to golden-brown, and were unbranced, flat or cured and smooth in shape, which had 1-3 septum transversums. Conidiophores (20-40×3-6 μm) had one single pore on the terminal and usually had slightly swollen basal-cells yielding conidium-chain. Conidia were from light-yellow-brown to golden-brown in colors, which are ovoid, upside down club, upside down pear or occasionally ellipse in shape, with pore on the basis. Ellipse conidia do not have beaks, or have short vertebra on the terminal without pore, or have club shape beaks. The club shape beaks is 2-4 μm in diameter, 10 μm for the longest, and is usually 1/4-1/3 length of conidia. Conidium, (10-)15-34×(5-)7-12 μm, had(1-)3-8 septum transversums, of which 1-6 cells were divided by 1-2 mediastina. On the base of conidiophore, one inclined septum is usually to divide the basal-cell and conidium-wall is smooth. Acropetal conidium-chain has 5 conidia (Neergaard 1945; Ellis 1971; Simmons 1992, 1993, 1995, 1998, 1999), (Fig. 1).

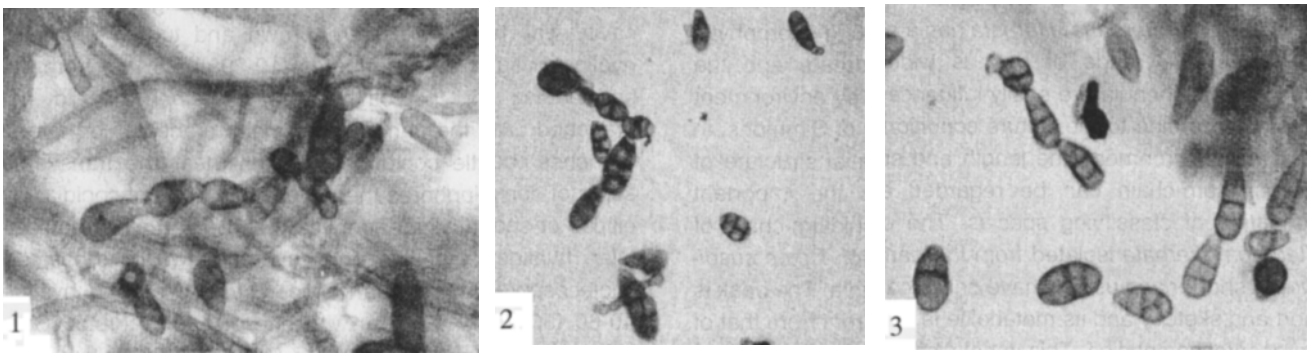

Fig. 1 ***Alternaria.alternata*** **(Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo**

1. Hyphae, conidiophores, proliferating conidium, and conidium-chain × 470;
2. Branching conidium-chain and beaklss (pseudorostrate) × 390;
3. Conidia and beakless (pseudorostrate) × 300.

Fig. 2 ***Phomopsis longiscoleospora*** **Y. Xiang et Lu An Guo,Wu Wen Fang**

1. Conidomata ×13;
2. Conidiophores and conidiogerenous cells' A-elipse or short cylinder conidia×190;
3. A-elipse or short cylinder conidia and B- scolecospores ×390.

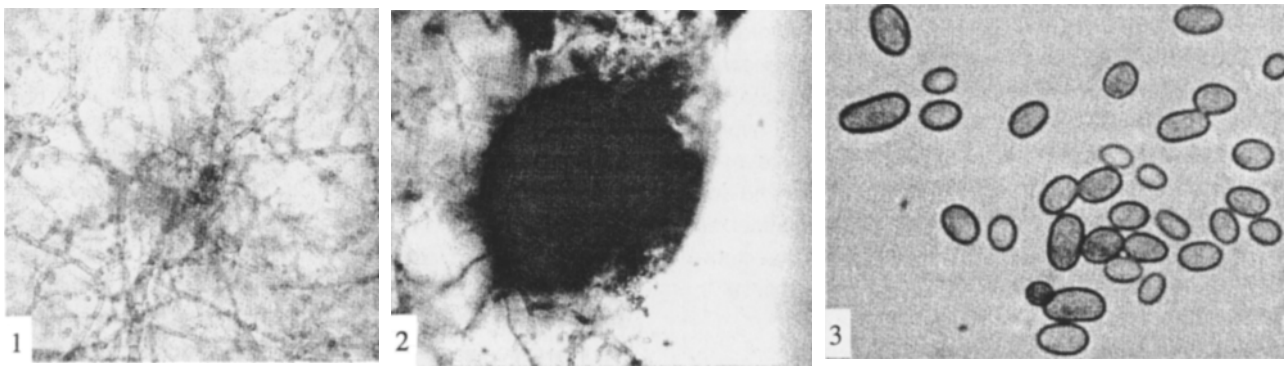

Fig. 3 ***Coniothyrium macrospoum*** **Y. Xiang et Lu An Guo,Wu Wen Fang**

1. Hyphal and process of new pycnidia in culture×140;
2. Pysnidia in culture×140;
3. Conidia×720.

Host and source: These strains were isolated from the bark of *Taxus cuspidata* in the Changbai Mountain Natural Reserve in Jilin province of China, in July 2000. Type specimens (HSSPD 1007, 1011, 1013) were preserved in Bioengineering and Biotechnology Pharmaceutical Teaching-Research Department of Shenyang Pharmaceutical University.

Discussion: *Alternaria alternata* has a strong saprophytic specificity. The range of host is widespread, and the shapes of its conndia are easily influenced by environment factors. According to the culture conditions of Simmons, in the same environment, the length and spatial structure of the conidium-chain can be regarded as the important foundation of classifying species. The conidium-chain of *Alternaria alternata* isolated from the bark of *Taxus cuspidata.* is shorter and usually have only 5 conidia. The beak is short and sketchy and its metabolite is different from that of representative species. This variety is firstly reported in the studies on Taxus.

(2) *Phomopsis longiscoleospora* Y. Xiang et Lu An Guo Wu Wen Fang sp. nov.

Characteristics: Colonies cultured on PDA at 25 °C were round, wooly, and radiate extension, and showed gray white in colors in initial stage and dust-colour on the reverse side in later stage. On the surface of colonies there were many globose or warty pycnida with a pore on apex, and yellow spore masses effused from pore. Mycelium was lightly brown to yellow brown. Ripe stromata with one pore and thick-wall, were brown in color, subglobose in shape, 1.5-2 mm in diameter and 0.5-1 mm in height. Stromata's cavity wall histo-cells were brown and turned gradually hyaline or light. Conidiophores (19-29×4-5.5 μm) from the surrounding of the cavity wall, branched or did not branched. At the terminal of main branches or lateral branches (bottle conidogenous cell) near the transverse septa of conidiophores there were two types of conidia: (1) ellipse or short cylinder conidia (6-9×2-2.4 μm), are unicellular, hyaline, with two ends tapering, two oil drops. (2) scolecospores are long-and-thin, curved unicellular, hyaline, 40-60 (50-70) ×1-1.5μm, with one or two oil drops (Saccardo 1884; Von Hohnel 1911; Grave 1935; Wollenweber *et al.* 1936, Sutton, 1980; Uecker 1988), (Fig. 2).

Host and source: These strains were isolated from the bark of *Taxus cuspidata*, in the Changbai Mountain Natural Reserve in Jilin Province of China, in July 2000. Type specimens (H1015, 1020) were preserved in Bioengineer-

ing and Biotechnology Pharmaceutical Teaching-Research Department of Shenyang Pharmaceutical University.

Discussion: Forty species in *Phomopsis* have been reported Their scolecospores were about 40 μm long and had one oil drop. Among them only one species *Phomopsis landeghemiae* (Sacc.) von Hohnelabout was found growing on the shoots of *Philadelphus coronaryus.*

(3) *Coniothyrium macrospora* Y. Xiang et Lu An Guo Wu Wen Fang sp. nov.

Characteristics: Flat colonies cultured on PDA at 25°C were gray in initial stage, and turned black gradually, with thick black particles and shiny surface. Septate mycelium was lightly brown, yellow brown to brown in the color. Pycnidia embedded or partly-embedded (160-276×69-161 μm) are broadly globose in the shape. On the apex of pycnidia, annellidic conidiogenous cells (4-9×3-5 μm) were ring-trace. Conidium with many patches on the surface were globose and ellipse or ovoid in the shape, and lightly yellow brown in the color (Saccardo 1884; Diedicke, 1912-1915; Archer 1926; Grave 1935; Biga *et al.* 1959; Sutton 1980) (Fig. 3).

Host and source: These strains were isolated from the bark of *Taxus cuspidata*, in the Changbai Mountain Natural Reserve in Jilin Province of China, in July 2000. Type specimens (H1026) were preserved in Bioengineering and Biotechnology Pharmaceutical Teaching-Research Department of Shenyang Pharmaceutical University.

Discussion: About 40 species have been reported in *Coniothrium*, but *Coniothyrium macrospora* Y. Xiang et Lu An Guo Wu Wen Fang sp. nov is first reported in this study to be live on *Taxus* so far. The conidia of this species are bigger, which is obviously different from the other members in the genus.

The other known species in China

(1) *Eurotium amsteloodomi* Mgngin. The anamorph is *Aspergillu vitis* Novor. (Tong1997) and the serial number of strain was 1001.

(2) *Eurotium repens* de Bary. The anamorph is *Aspergillus reptans* Samson & Gams. (Tong 1997) and the serial number of strain was 1003.

(3) *Botrytis* sp. (Barnett *et al.* 1972; von Arx., 1974). The serial number of strain was 1014, 1021 and 1027.

(4) *Penicillium citrinum* Thom (John 1979). The serial number of strain was 1018.

(5) *Epicoccum nigrium* LinK. (Wei 1979). The serial number of strain was 1024.

(6) *Fusarium* sp. (Barnett *et al.* 1972; von Arx., 1974). The serial number of strain was 1017.

Screen of strains

(1) The strains that theirs fermentation metabolites reacted positively with taxoids developer - the vanillic aldehyde were *Eurotium amstelodomi* Mgngin 1001, *Eurotium repens* de Bary 1003, *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo 1011, and *Botrytis* sp.1014 in turn.

(2) The strain that theirs fermentation metabolites reacted positively with dilute bismuth potassium iodide was *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo 1011.

The determination of Target strain

Through the screen of all the above strains, the metabolites of *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo 1011 were steady. A component of the fermentation metabolites of *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo 1011 could react with the vanillic aldehyde to show purplish red in the color and its content is more. This component had a UV absorption under 253.7 nm and 365.0 nm, so it is easy to differentiation and collection. This component also can react with the dilute bismuth potassium iodide to show purplish red in the color, and this strain was determined as target strain.

The structure of the fermentation metabolite of target strain

The strain (*Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo Wu Wen Fang 1011) was screened by studying the strains' metabolites. The compound produced by *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo 1011, was separated, purified from its liquid fermentation metabolites and was named compound I. Through spectrum Analysis, ultraviolet scanning, vanillic aldehyde developing, dilute bismuth potassium iodide (alkaloids developer) developing, the chemical structure of compound I was identified as one kind of taxiods type III. The chemical name of compound I is: 2-oxygen ethyl-5-ethylamine-7, 10- dihydroxy-20- [4-2,6 (oxygen ethyl-benzene)-1,3-diene]-4,20-diene-paclixtel-11-monoen oic -9, 13-diketon (Fig. 1, Fig. 2). The above results show that the strain can highly produce taxoids.

Discussion

According to literatures, the distribution of endophytic fungi in their hosts is affected by many factors, such as, the age of trees, the season, the height above sea level, and the number of specimens. The species number of endophytic fungi in their hosts is also different. In order to acquire more useful endophytic fungi, more collection places and sites should be added, including trunks, main branches, slight branches and so on, thus obtaining more strains of endophytic fungi.

The strains of endophytic fungi are easy to present variation as increasing of subculture times. For preventing aging and degeneration of strains, it is important to control culture times, reduce times of subculture, and to do well in isolation, rejuvenescence and preservation.

The specimens for isolation are usually contaminated by the other germs living in the air and on the surfaces of the plants. In order to get rid of other useless germs and insure

the purity of endophytic fungi of the host, we added the bacterial inhibitor to the medium, and sampled from the inside of the specimens that have been disinfected to the utmost.

Nineteen endophytic fungi were isolated from *Taxus cuspidata*, besides two of them were ascomycetes, and the others were deuteromycete, or hyphomycete.

The strain *Alternaria alternata* (Fr.) Keissler var. *taxi* Y. Xiang et Lu An Guo, 1011 isolated from the bark of more than 200-year-old *Taxus cuspidata* in the primeval forest of the Changbai Mountain Natural Reserve, can produces taxiods of type III. The reported compound and taxiods of type I (paclitaxel) are all taxiods, and its antineoplastic action should be further studied.

References

Ainsworth, G.C. Sparrow, F.K. and Sussman, A .S. 1973. The Fuyi An advanced tretise 4A [M]. New York and London: Academic Press, p1-621.

Archer, W.A. 1926. Morphological characters of some *Sphaeropsidales* in culture [J]. Ann. Mycl., **24**: 1-80.

Barnett, H.L. and Barry, B. 1972. Illustrated genera of imperfect fungi (3rd ed) [M]. Minneepolis, Minnesota: Burgess Pub. Com., p241.

Biga, M.L.B., Cifrri, R. and Bestagno, G. 1959. Ordinamento artificialle delle Specie del genere Coniothyrium Corda [J]. Sydowia, **12**: 248-325.

Diedicke, H. 1912-1915. Kryptogamenflora der Mark Brandenburg, IX Pilze VII, *Sphaeropsideae, Melanconieae* [M]. Leipzig: Borntraegr bros., p1-460.

Ellis, M.B., 1971. Dematiaceous Hyphomycetes [M]. England: C.M.I., Kew, Surry., p1-608.

Fang Zhongda. 1979. The study methods of plant disease [M]. Beijing: Agriculture Publishing Company, p102-160. (in Chinese)

Fisher, P.J., Petrini, O. and Sutton, B.C. 1993. A comparative study of fungal endophytes in leaves,xylem and bark of *Eucalyptus nitens* in Australia and England [J]. Sydowia, **45**: 338-345.

Grave, W.B. 1935. Brtish stem and leaf fungi (Coelomycetes) [M]. London and New York: Cambridge Univehsity Press, Vol. 1, p. 205.

Grave, W.B. 1935. British stem and leaf fungi (Coelonuycetes) [M]. London and New York: Cambridge University Press, Vol. 1, p451-45.

Guo Liangdong. 2001. Studies progression of endophytic fungi [J]. Mycology System, **20** (1): 148-152. (in Chinese)

Helander, M.L., Neuvonen S., Sieber T., *et al.* 1993. Simulated acid rain affets birch leaf endophyte populations [J]. Microb Ecol., **26**: 227-234.

Wei Jingchao. 1979. Fungi identification manual [M]. ShangHai: Shangai Technology Press Inc., p641. (in Chinese)

John, I.P. 1979. The enus *Penicllium* and its teleomorphic states *Eupenicillium* and *Talaromyces* [M]. London LTD: Academic Press Inc., p1-654

Neergaard, P. 1945. Donish spedies of *Alternaria* and *Stemphylium* [M]. Copenhagen: E. Munksgaard, p1-560.

Saccardo, P.A. 1884. Sylloge fuugorum omnium hucusque cognitorum [M]. Italy: Pavia, Vol. 3, 1-860.

Simmons, E.G. 1967. Typification of Alternaria, Stemphylium and Ulocladium, [J]. Mycologia, **59**:73-74.

Simmons, E.G. 1992. Alternaria taxonomy: current status, viewpoint, challenge [C]. In: Chelkowki, J. and Visconti, A. (eds.), Alternaria Biology Plant Disease and Amsterdam: Elsevier, 1-29.

Simmons, E.G. 1993. Alternaria themas and variations (73) [J]. Mmycetaxon, **48**:109-140.

Simmons, E.G. 1995. Alternaria themas and variations (113-144) [J]. Mmycetaxon, **55**: 55-163.

Simmons, E.G. 1998. Alternaria themas and variations (224-225) [J]. Mmycetaxon, **68**: 417-427.

Simmons, E.G. 1999. Alternaria themas and variations (226-243) [J]. Mmycetaxon, **70**: 263-36.

Stobel G, Yang X. S., Sears J., *et al.* 1996. Taxol from *Pestalotiopsis microspora* an endophytic fungus of *Taxus wallachiana* [J] Microbiology, **142**: 435-440.

Sutton, B.C. 1980. The Coelomycetes [M]. Englond: C.M.I.,Kew, Surrey., p1-696.

Uecker, F.A. 1988. A world list of *Phomopsis* names with on nomenclature, morphology and biology [J]. Mycol. Mem., **13**: 1-231.

Von Arx. 1974. The genera of fungi sporulating in pure culture[M]. LEHRE: VERIAG von C ramer, 1-315.

Von Hohnel, F. 1911. Zur Systematikder Sphaeropsideen und Melanconieen [J]. Ann. Mycol., **9**: 258-265.

Wollenweber, H.W., and Hochapfel. H. 1936. Beirage zur Kenntnis parasitarer und sapro-phytischer pilze.I. *Phomopsis, Dendrophoma* und *Ascochyta* und ihre Bezeihung zur Fruchtfule [J]. Z. Parasitenk, **8**: 561-605.

Xiang Yong, Cui Jingxia and Lu Anguo. 2002. Isolation and screen of Taxus cuspidata Sieb. et Zucc. endophytic fungi [J]. Journal of Northeast Forestry University, **30** (2): 30-34. (in Chinese)

Xiang Yong, Liu Jun, Lu Anguo, *et al.* 2003. Separation purification and structure identification of taxoids-produced endophytic fungi Alternaria alternata var. *taxi* 1011 [J]. Journal of Forestry Research, **14**(3): 217-220.

Qi Zutong. 1997. Volume 5 of China fungi-Aspergillus and its relatedte teleomorph [M]. Beijing: Science Press Inc., 134-136, 145-147. (in Chinese)